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Major Heat-Modifiable Outer Membrane Protein in Gram-Negative Bacteria: Comparison with the OmpA Protein of *Escherichia coli*

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The outer membranes of several strains of *Escherichia coli*, other enteric bacteria, and a variety of nonenteric gram-negative bacteria all contain a major heat-modifiable protein similar to the OmpA protein of *E. coli* K-12. The heat-modifiable proteins from these bacteria resemble the K-12 protein in molecular weight, in preferential release from the outer membrane by sodium dodecyl sulfate in the presence of Mg^{2+}, and in characteristic cleavage by proteases to yield a smaller fragment which remains membrane bound. Antiserum directed against the K-12 protein precipitated the heat-modifiable protein from all strains of *Enterobacteriaceae*, and chemical comparison by isoelectric focusing, cyanogen bromide cleavage profiles, and proteolytic peptide analysis indicated that the proteins from the various enteric bacteria were nearly identical in primary structure. The heat-modifiable proteins from bacteria phylogenically distant from *E. coli* shared many of the properties of the *E. coli* protein but were chemically distinct. Thus, it appears that the structure (and, presumably, the function) of the heat-modifiable protein of gram-negative bacteria is strongly conserved during evolution.

The OmpA protein of *Escherichia coli* K-12 serves as a cell surface receptor for certain bacteriophage and colicins (6, 12, 16), and mutants resistant to these agents lack the OmpA protein (5, 9, 16). Such mutants demonstrate a defect in ability to accumulate specific low-molecular-weight solutes, grow poorly at temperatures above 37°C, and rapidly lose viability in the stationary phase (1, 12, 16, 36). Mutants missing both the OmpA protein and the murein lipoprotein are not viable (33). The most reasonable interpretation of these results is that the OmpA protein is important for maintenance of the outer membrane integrity (16, 33). This same mutation also causes the inability of the bacteria to act as recipients in conjugation with F^+ donors, indicating a more specific role for the OmpA protein in conjugation (31, 37).

The OmpA protein has several unique characteristics which distinguish it from the other outer membrane proteins of *E. coli* K-12 (8, 21, 27, 37). When the protein is heated to 100°C, in the presence of anionic detergents such as sodium dodecyl sulfate (SDS), the OmpA protein undergoes a characteristic conformational change (29, 31). This heat modifiability raises the apparent molecular weight from 28,000 to 33,500. The OmpA protein is characteristically released from the peptidoglycan by extraction with SDS in the presence of Mg^{2+} at temperatures below 56°C and is readily cleaved by treating the outer membrane with proteases (28, 32, 37).

The importance of OmpA protein in the outer membrane of *E. coli* K-12 suggested that a structurally and chemically similar protein might be present in the outer membrane of other gram-negative bacteria. Consistent with this idea, a protein similar to the OmpA protein of *E. coli* K-12 has been identified in several species of gram-negative bacteria (6, 7, 10, 17, 18, 30). We have studied a variety of gram-negative bacteria and found that all of them have an OmpA-like protein present in the outer membrane. A detailed chemical comparison indicates that the primary structures of these heat-modifiable proteins differ with phylogenetic distance from *E. coli* K-12.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and culture media. The *E. coli* K-12 strain used was CS109 (W1485F$^-$, 23). The other *E. coli* strains were clinical isolates N71-S1 (serotype O78), N7-S10 (serotype O9), and N24-S1 (serotype O16/O62) from Kenneth Vosti, Stanford University Medical School. *Salmonella typhimurium* LT-2 strain SH5014 (*his metA metE trp ilv thr xyl flaA rfaJ rpsL*) was from Helena Mäkelä, Helsinki, Finland. *Haemophilus influenzae* CDC76-646 and *Neisseria gonorrhoeae* $GC8_2$ were from Owen

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Hendley, University of Virginia Medical School. *Erwinia carotovora* ICPB EC153 was from Mortimer Starr, University of California at Davis. *Shigella sonnei*, *Shigella boydii*, and *Klebsiella pneumoniae* were clinical isolates from our departmental culture collection, and the remainder of the organisms listed in Table 1 were clinical isolates supplied by the clinical laboratories of the University of Virginia Hospital. The Hfr strain was CS777 (KL208, *pyrD ompA relA1*) and carried the transposon Tn*10* 60% cotransducible with *pyrD* and *ompA*.

Bacteriophages were K3 and its host range mutants K3h1 and K3h4 from Paul A. Manning and Peter Reeves (9, 16), Ox2 (9) from Peter Reeves, and TuII* (33) from Ulf Henning via John Foulds. Bacteriophage sensitivity was determined by the cross-streak method or by spotting dilutions of the phages onto lawns of the indicator strains (24).

Culture media were tryptone-yeast extract broth and minimal salts medium A as used previously (24). *H. influenzae* and *N. gonorrhoeae* were grown in GC medium (11) supplemented before use with 2% IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.). Minimal medium was supplemented with the appropriate growth factors (see above) at 100 μg/ml. Glucose was used as the energy source (0.5%) in minimal medium for all strains except *Pseudomonas* spp., for which 0.25% sodium gluconate was used. For the preparation of labeled cells, we used medium A containing 0.1 μCi of [^{14}C]leucine or 0.8 μCi of [^{3}H]leucine per ml together with 8 μg of carrier leucine per ml. Broth cultures were grown with good aeration at 37°C, except for *E. carotovora*, which was grown at 30°C. Cells were harvested when the density reached 6×10^8 to 8×10^8 cells per ml.

Purification of proteins. The basic procedures used for purifying Triton-insoluble proteins by anion-exchange chromatography and gel filtration are described elsewhere (15). Harvested cells were first broken in a French pressure cell, and isolated membranes were extracted with 2% Triton X-100 to remove the Triton-soluble fraction. The Triton-insoluble wall was then extracted with 1% SDS in the presence of 100 μM $MgCl_2$ as described previously (32). The soluble frac-

TABLE 1. *Characterization of the major heat-modifiable outer membrane proteins from gram-negative bacteria*

Strain	Apparent mol wt[a] (10^3)		Cleaved by trypsin[b]	Apparent mol wt[a] of trypsin fragment (10^3)	Sensitivity to OmpA-specific phage	Shared antigenicity[c] with OmpA protein
	Unheated	Heated				
Escherichia coli K-12	28	33.5	+	24	All	+++
E. coli N71-S1	28	33.5	+	24	Some	+++
E. coli N7-S10	28	34	+	24	−	+++
E. coli N24-S1	28	34	+	24	Some	+++
Shigella sonnei	28	33.5	+	24	All	+++
S. boydii	28	33.5	NT[d]	NT	All	NT
Salmonella typhimurium LT2	28	33	+	24	Some	++
Citrobacter freundii	28	35	+	26	Some	++
Klebsiella pneumoniae	28	35	+	25	−	+
Enterobacter aerogenes	28	35	+	25	−	+
Proteus vulgaris	31	38	+	NR[e]	−	+
P. mirabilis	33	37	NT	NT	−	NT
Serratia marcescens	28	34	+	27	−	+
Providencia alcalifaciens	31	37	+	24	−	+
Erwinia carotovora	28	36	+	NR	−	+
Haemophilus influenzae	28.5	35	+	26	−	−
Moraxella sp.	32	37	+	NR	−	−
Neisseria gonorrhoeae	23, 25	30, 31	+, +	NR	−	−
Vibrio parahaemolyticus	29	35	NT	NT	−	NT
Pasteurella multocida	27	35	NT	NT	−	NT
Yersinia enterolytica	29	35	NT	NT	−	NT
Pseudomonas aeruginosa	37	42	+	NR	−	−
P. fluorescens	37	42	NT	NT	−	NT

[a] As determined by the Tris-glycine-buffered SDS-gel system with bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (29,000), egg lysozyme (14,300) and myoglobin (17,300) as molecular weight standards.

[b] Isolated Triton-insoluble envelope (1 mg of protein per ml) was incubated for 30 min at 37°C with 100 μg of trypsin per ml. The mixture was then heated to 100°C in sample buffer for gel electrophoresis.

[c] Antigenic cross-reactivity was determined by quantitation of the protein precipitated directed against the K-12 OmpA protein and resolved by SDS-gel electrophoresis, and by titration of the antiserum-antigen reaction in double-diffusion assays.

[d] NT, Not tested.

[e] NR, Not resolved in SDS-gels.

tion obtained after centrifugation for 1 h at 46,000 × *g* contained the major heat-modifiable protein. This fraction was further purified as described previously (15), and the final product was the major heat-modifiable protein substantially free from contaminating proteins. In some cases, the major heat-modifiable protein was purified from the SDS-Mg^{2+}-soluble extract by polyacrylamide gel electrophoresis, using the Tris-glycine buffer system (see below). The material was dissolved in sample buffer at 37°C, and the gels (3 mm thick) were run in the normal way. The major heat-modifiable protein was located by staining a strip cut from the edge of the gel. The appropriate band was then cut from the remaining frozen gel, and the protein was eluted by mascerating the gel and washing it with 50 mM Tris-hydrochloride (pH 7.2) containing 0.1% SDS until no further protein was released. Control experiments, in which *E. coli* K-12 OmpA proteins purified by elution from gels and by anion-exchange chromatography and gel filtration, were compared by cleaving with proteases and comparing the peptide profiles obtained by high-pressure column chromatography (see below), demonstrated that proteins purified by the two methods were indistinguishable.

In all cases, the final protein preparation was heated to 100°C in 1% SDS for 5 min, concentrated by filtration through an Amicon PM10 membrane filter, and precipitated with 5 volumes of cold acetone at −70°C. Acetone precipitation was repeated twice, and the proteins were suspended in distilled water and lyophilized.

Polyacrylamide gel electrophoresis. The three different systems used for resolving proteins by polyacrylamide gel electrophoresis in the presence of SDS were all as described previously (15, 35). In the case of electrophoresis used to resolve proteins in smooth strains of bacteria, the concentration of SDS in the upper buffer was increased to 0.15 to 0.25% (35).

Isoelectric focusing. The procedure used for isoelectric focusing was the previously published modification (15) of the technique developed by O'Farrel (23), except that tube gels were used with Bio-Rad pH3-10 ampholytes (Bio-Rad, Richmond, Calif.). After focusing, the gels were frozen, cut into 1.3-mm slices, and counted for radioactivity as described previously (15).

Peptide analysis. Acetone-precipitated protein samples were digested with trypsin plus chymotrypsin as previously described (15), and the peptides were resolved by high-pressure chromatography on Aminex A-6 resin (15). Since peptide elution profiles are not identical from run to run, all comparisons were made by mixing ^{14}C- and ^{3}H-labeled samples before protease digestion and then cleaving and analyzing the mixture.

Cleavage with CNBr was performed as described previously, using a 70% formic acid or 70% trifluoroacetic acid solvent. Proteins labeled with [^{14}C]leucine or [^{3}H]leucine were digested simultaneously to ensure reproducibility. Cleaved peptides were separated by SDS-gel electrophoresis, using the cacodylate-phosphate gel system (15; A. P. Pugsley and C. A. Schnaitman, Biochim. Biophys. Acta, in press).

Immune precipitation. Antiserum was prepared against unheated OmpA protein purified from the outer membrane of *E. coli* K-12 strain CS109, using the regimen described previously (36). The primary injection was given intradermally instead of in the footpads.

Double-diffusion analysis was carried out in 1% agarose containing 50 mM Tris-hydrochloride (pH 7.2), 1% Triton X-100, and 50 mM NaCl (TTS). Outer membrane protein was solubilized by suspension of the Triton-insoluble envelope fraction in TTS, incubation at 37°C for 30 min, and centrifugation for 30 min at 46,000 × *g* to remove insoluble material. The outer wells contained TTS-soluble material at 1 mg/ml, and the center well contained antiserum specific for *E. coli* K-12 OmpA protein. Immune precipitation was carried out as previously described (36), and the protein was analyzed by SDS-gel electrophoresis, using the Tris-glycine buffer system (A. P. Pugsley and C. A. Schnaitman, in press).

Conjugations. Conjugations with Hfr strain CS777 and streptomycin-resistant derivatives of clinical *E. coli* isolates were performed in tryptone-yeast extract broth for 2 h with a 1:5 ratio of donors to recipients. Transcipients were selected by plating on medium containing 15 μg of tetracycline per ml and 100 μg of streptomycin per ml.

RESULTS

Primary identification of a major heat-modifiable protein. A major heat-modifiable protein was detected in the outer membrane fraction of all 26 smooth strains of *E. coli* examined, as well as in *E. coli* B. The protein accounted for approximately 20% of the total outer membrane protein in all cases except in *E. coli* B, which produced less heat-modifiable protein (2). Furthermore, the heat-modifiable protein from each strain comigrated with the K-12 OmpA protein in SDS-gel electrophoresis when examined in the unheated form (apparent molecular weight, 28,000). There was some variability in the apparent molecular weight of these proteins (33,000 to 35,000) after boiling in SDS (Table 1, Fig. 1). A major heat-modifiable protein was also detected in the outer membrane fraction of all the other gram-negative bacteria studied (Fig. 2). In these genera, there was variation in the relative migrations of both the heated and unheated forms of the protein in SDS-gels (Fig. 2, Table 1). It was noted that in the case of *N. gonorrhoeae*, two major heat-modifiable proteins were resolved (Fig. 2).

Since the major heat-modifiable proteins in the outer membrane fraction of all the bacteria studied were similar with respect to migration in SDS-gels, it was of interest to determine whether they shared other physical properties with the OmpA protein of *E. coli* K-12. Treatment of isolated outer membranes with SDS plus Mg^{2+} at 56°C resulted in the preferential release of the heat-modifiable protein in all cases except *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*. Furthermore, in each case in

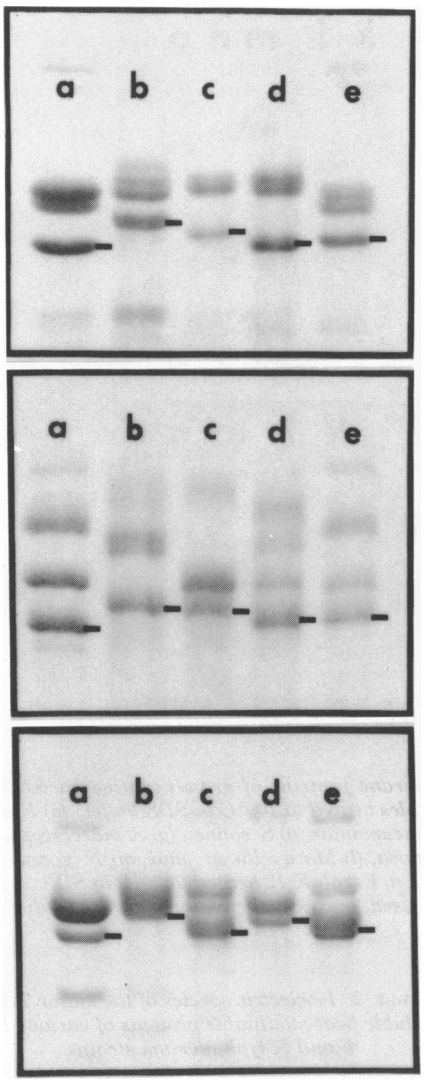

FIG. 1. *SDS-gel electrophoresis of the major Triton-insoluble proteins of E. coli K-12 (a), S. typhimurium LT2 (b), E. coli N71-S1 (c), E. coli N7-S10 (d), and E. coli N24-S1 (e). All samples were heated to 100°C in SDS sample buffer before electrophoresis. Top, Tris-glycine buffer system; center, Tris-glycine buffer with 8 M urea; bottom, phosphate-cacodylate buffer system. The major heat-modifiable proteins are indicated by dashes. Only the regions of the gels displaying the proteins of molecular weights 28,000 to 50,000 are shown.*

which it was tested, the heat-modifiable protein was preferentially cleaved with trypsin under conditions which led to cleavage of the K-12 OmpA protein to a 24,000-molecular-weight membrane-bound fragment (Table 1).

Genetic evidence supporting a direct relationship between the heat-modifiable proteins for *E. coli* K-12, *S. typhimurium* LT-2, and the smooth *E. coli* strains was further established. In the case of *S. typhimurium*, mutants resistant to bacteriophage Ox2, which utilizes the K-12 OmpA protein as its receptor on the cell surface, lacked the heat-modifiable protein from the outer membrane as determined by SDS-gel electrophoresis. These strains presumably carry mutations within the *S. typhimurium ompA* gene (34). Clinical *E. coli* isolates were converted to *ompA* by conjugating with Hfr strain CS777, which transfers transposon Tn*10* and the K-12 *ompA* mutation at high frequency, indicating that the heat-modifiable protein produced in these strains had the same structural gene locus as K-12.

Chemical and serological comparisons of the major heat-modifiable proteins. Purified major heat-modifiable proteins from three strains of *E. coli* and one strain of *S. typhimurium* were compared with the K-12 OmpA protein by isoelectric focusing, SDS-gel electrophoresis of cyanogen bromide cleavage fragments, and high-pressure liquid chromatography of protease peptides. The two isoelectric species of each of the four proteins had isoelectric points that were similar to, but reproducibly different from, those of *E. coli* K-12 (Fig. 3, Table 2). Profiles obtained by SDS-gel electrophoresis of cyanogen bromide-cleaved proteins were also nearly identical, with small differences seen for the electrophoretic mobilities of some peptides (not shown).

The most detailed evidence for the overall similarity of these proteins was provided by high-pressure liquid chromatographic examination of the peptides produced by protease cleavage of leucine-labeled proteins. When the heat-modifiable proteins from the various *E. coli* strains were cleaved simultaneously with OmpA protein from *E. coli* K-12, the elution profiles of the peptides were identical or nearly identical (an example is shown in Fig. 4A). The major heat-modifiable protein of *S. typhimurium* LT2 was also nearly identical to the OmpA protein of *E. coli* K-12 by this criterion (Fig. 4B).

Similar results were obtained for the major heat-modifiable proteins from other genera which were sensitive to phage K3, K3h1, or Ox2. For example, high-pressure liquid chromatography of protease-digested *S. sonnei* protein and the K-12 OmpA protein had identical peptide profiles (not shown), and the peptide profile obtained after cleavage of the *Citrobacter freundii* major heat-modifiable protein was nearly identical to that obtained for the K-12 OmpA protein (not shown).

Chromatography of protease-digested, heat-modifiable protein from *K. pneumoniae* (Fig.

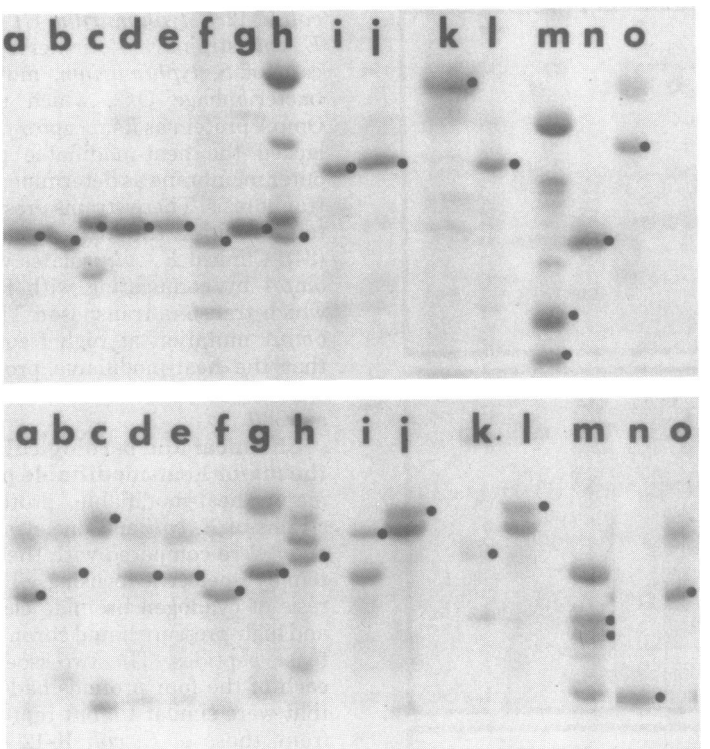

FIG. 2. *SDS-gel electrophoresis of Triton-insoluble membrane proteins of gram-negative bacteria. Top, Samples heated to 30°C in SDS sample buffer. Bottom, Samples heated to 100°C in SDS buffer: (a) E. coli K-12, (b) C. freundii, (c) E. carotovora, (d) E. aerogenes, (e) K. pneumoniae, (f) S. sonnei, (g) S. marcescens, (h) H. influenzae, (i) P. alcalifaciens, (j) P. vulgaris, (k) P. aeruginosa, (l) Moraxella sp., and (m) N. gonorrhoeae. Samples n and o are the same in both top and bottom panels; n, E. coli K-12 heated to 30°C in SDS; o, E. coli heated to 100°C in SDS. The Tris-glycine buffer system was used, and only the regions of the gels displaying the major heat-modifiable proteins are shown.*

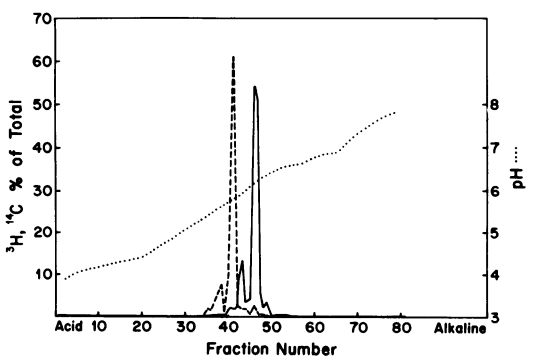

FIG. 3. *Isoelectric focusing of purified heat-modifiable protein from E. coli strains K-12 (—, [^{14}C]-leucine labeled) and N7-S10 (- - -, [^{3}H]leucine labeled).*

TABLE 2. *Isoelectric species of the major Triton-insoluble heat-modifiable proteins of various E. coli and S. typhimurium strains*

Protein from strain[a]	Isoelectric species in order of abundance
Escherichia coli K-12 CS109	5.75 (75), 5.5 (25)
E. coli N71-S1	5.4 (66), 5.2 (34)
E. coli N7-S10	5.35 (70), 5.2 (30)
E. coli N24-S1	5.5 (76), 5.35 (24)
Salmonella typhimurium SH5014	5.2 (70), 5.5 (30)

[a] Samples were [^{3}H]leucine- or [^{14}C]leucine-labeled proteins, and contained the *E. coli* K-12 OmpA protein from CS109 with one other protein with the opposite label. Figures indicate pI of the isoelectric species and numbers in parentheses are percentages of total counts recovered from the sliced gel.

4C), *Enterobacter aerogenes* (not shown), and *Providencia alcalifaciens* (not shown) produced peptide profiles which were similar to that obtained after cleavage of the K-12 OmpA protein. However, there was clearly less similarity between each of these profiles and that of *E. coli* K-12 as compared with those obtained with the smooth *E. coli* strains. The major heat-modifiable proteins of *Serratia marcescens* and *Proteus vulgaris* were even less closely related to the K-12 OmpA protein but still demonstrated

a high degree of peptide similarity (not shown). The peptide profiles obtained by high-pressure chromatography after cleavage of *E. carotovora* and *P. aeruginosa* were quite different from the peptide profile of the *E. coli* K-12 OmpA protein (Fig. 4D and E).

Evidence for a relationship between the heat-modifiable proteins of the different *E. coli* strains was further provided by the observation that antiserum against highly purified, unheated *E. coli* K-12 OmpA protein could precipitate the heat-modifiable protein from solubilized preparations of outer membrane fractions from these strains (Table 1). When these immune precipitates were examined by SDS-gel electrophoresis, only the major heat-modifiable protein was detected (not shown). Antiserum against the K-12 OmpA protein also precipitated the heat-modi-

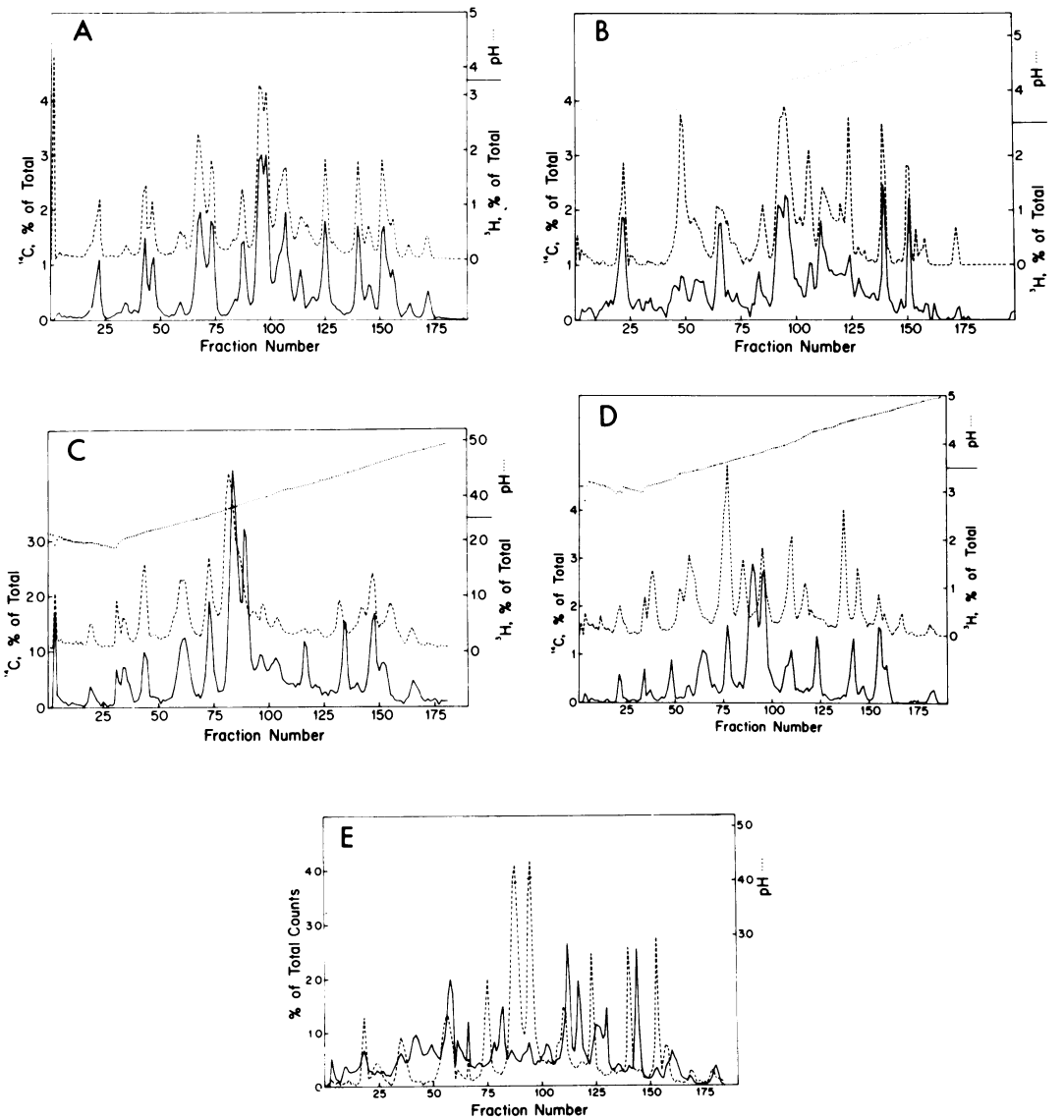

FIG. 4. *Proteolytic cleavage peptide profiles of major Triton-insoluble heat-modifiable proteins digested with trypsin plus chymotrypsin. (A) E. coli K-12 OmpA protein (—, [^{14}C]leucine) and E. coli N7-S10 heat-modifiable protein (- - -, [^{3}H]leucine); (B) E. coli K-12 OmpA protein (·····, [^{3}H]leucine) and S. typhimurium LT2 heat-modifiable protein (—, [^{14}C]leucine); (C) E. coli K-12 OmpA protein (—, [^{14}C]leucine) and K. pneumoniae heat-modifiable protein (·····, [^{3}H]leucine); (D) E. coli K-12 OmpA protein (—, [^{14}C]leucine) and E. carotovora heat-modifiable protein (·····, [^{3}H]leucine); (E) E. coli K-12 OmpA protein (·····, [^{3}H]leucine) and P. aeruginosa heat-modifiable protein (—, [^{14}C]leucine).*

fiable protein from genera within the family *Enterobacteriaceae*, although the extent of cross-reactivity decreased with the decreased relatedness to *E. coli* K-12. It appears that a direct relationship exists between the extent of antigenic cross-reactivity of the heat-modifiable proteins from the *Enterobacteriaceae* and the extent of structural similarity as determined by high-pressure liquid chromatography of protease-derived peptide profiles (Table 1).

DISCUSSION

There are three classes of proteins which are very abundant in the outer membranes of enteric bacteria: the murein lipoprotein, the porins or transmembrane pore proteins (19, 36), and the heat-modifiable or OmpA protein. The lipoprotein is the most abundant outer membrane protein and is probably present in all gram-negative bacteria (19, 22). This protein appears to be conserved during evolution, since there is evidence that lipoproteins from different genera of bacteria may have substantial sequence homology (22). In contrast, the porin proteins do not appear to be conserved during evolution even though all gram-negative bacteria must have a functional equivalent of these proteins. The porin proteins exhibit little homology either within a species or between different genera and species. The OmpC and OmpF porin proteins which are both produced by *E. coli* K-12 appear to have evolved from a common ancestral gene, yet these very similar proteins have almost no identical proteolytic peptides (15). We have recently compared the OmpC and OmpF porin proteins of *E. coli* K-12 with the genetically equivalent proteins produced by *S. typhimurium* LT2, and found that less than half of the proteolytic peptides between the same proteins produced by these two closely related species (14) were identical.

The heat-modifiable or OmpA protein resembles the lipoprotein in that it is strongly conserved during evolution. When the OmpA protein of *E. coli* K-12 was compared to the same protein from unrelated *E. coli* strains or *S. typhimurium*, minor differences in electrophoretic mobility were observed. However, the proteins from these strains had almost identical proteolytic peptide profiles and cross-reacted with antiserum to the K-12 OmpA protein, and all of these strains exhibited some sensitivity to OmpA-specific phage. Each of these strains had a single genetic locus determining the production of the protein which was equivalent to the OmpA locus of *E. coli* K-12. This is also in contrast to the porin proteins, which in some cases have multiple genetic loci coding for identical or similar proteins (15, 25; A. P. Pugsley and C. A. Schnaitman, Mol. Gen. Genet., in press).

Among the more distantly related members of the *Enterobacteriaceae*, one can construct a phylogenetic tree based on similarity of the heat-modifiable proteins which is similar to those already proposed from studies with murein lipoprotein, alkaline phosphatase, or 5S rRNA (4, 15, 23). All of the gram-negative bacteria tested had one or more outer membrane proteins which exhibited a heat-modifiable behavior similar to that of the *E. coli* OmpA protein, and in some cases it was also possible to demonstrate partial trypsin cleavage similar to that observed with the *E. coli* protein. Thus, even in those distantly related organisms where peptide profiles are no longer similar, it is likely that the overall domain structure leading to heat modifiability and unique tryptic cleavage has been conserved. Thus, it appears that most, if not all, gram-negative bacteria have a protein which is structurally (and, presumably, functionally) similar to the OmpA protein of *E. coli*.

It can be argued that the rapid evolution observed in the porin proteins of the enteric bacteria reflects the fact that these proteins are exposed on the cell surface and function as receptors for bacteriophages, bacteriocins, or other lethal agents. However, the OmpA protein of *E. coli* also functions as a receptor for several phages, indicating that it too is exposed on the cell surface and subject to the same selective pressures. Thus, the conservation of structure of the OmpA protein as compared to the porin proteins suggests that, although both classes of proteins perform essential functions for the cell, only minor changes in the amino acid sequence of the OmpA protein can be tolerated without loss of function. This would be consistent with the proposed role of the OmpA protein as a structural or organizational component of the outer membrane, since in such a role the protein might have to interact in a specific manner with a number of other membrane components.

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